Research Article | Vol. 9, No. 4 | 1 Apr 2018 | BIOMEDICAL OPTICS EXPRESS 1783
Biomedical Optics EXPRESS

Technique of laser chromosome welding for chromosome repair and artificial chromosome creation

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Abstract: Here we report a technique of laser chromosome welding that uses a violet pulse laser micro-beam for welding. The technique can integrate any size of a desired chromosome fragment into recipient chromosomes by combining with other techniques of laser chromosome manipulation such as chromosome cutting, moving, and stretching. We demonstrated that our method could perform chromosomal modifications with high precision, speed and ease of use in the absence of restriction enzymes, DNA ligases and DNA polymerases. Unlike the conventional methods such as de novo artificial chromosome synthesis, our method has no limitation on the size of the inserted chromosome fragment. The inserted DNA size can be precisely defined and the processed chromosome can retain its intrinsic structure and integrity. Therefore, our technique provides a high quality alternative approach to directed genetic recombination, and can be used for chromosomal repair, removal of defects and artificial chromosome creation. The technique may also have applicability on the manipulation and extension of large pieces of synthetic DNA.

OCIS codes: (140.0140) Lasers and laser optics; (170.0170) Medical optics and biotechnology.

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#319841 https://doi.org/10.1364/BOE.9.001783
Journal © 2018 Received 22 Jan 2018; revised 5 Mar 2018; accepted 6 Mar 2018; published 21 Mar 2018

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1. Introduction

Recently, the micromanipulation of a single chromosome or chromosome fragment has attracted much attention due to its potential application in genomic sequencing, genetic engineering, DNA or chromosome repair and artificial chromosome creation [1–5]. There are a variety of methods available for chromosome micromanipulation [6–13], but the conventional methods have inherent limitations. For example, in the techniques of chromosome engineering/modification, since each type of restriction enzyme only recognizes a specific nucleotide sequence for DNA cutting, specific enzymes and sequences are needed to modify a chromosome, existing techniques usually require a substantial amount of time to develop the enzymes. The techniques also have a number of challenges including the difficulty to control and access target chromosomes, and the inadequacy to repair or remove defective chromosomes. Therefore, alternative approaches to genetic material micromanipulation are desirable.

The purpose of the present paper is to overcome the shortcomings and deficiencies of existing technologies to enable modification of genetic material without requiring the use of restriction enzymes for chromosome cutting, and DNA ligases and polymerases for chromosome connection. As a direct and intended consequence, chromosomes can be modified at any desired location rather than limited to the site specificity of a particular restriction enzyme or DNA ligases by applying laser micro-beam techniques to micro-manipulate chromosomes to a fine degree of control.

In our method, the chromosome is processed and manipulated to effect a desired chromosomal change by utilizing the technique of laser chromosome welding in combination with laser chromosome cutting and moving.

The technique of chromosome cutting was developed about twenty years ago and performed using a micro-beam laser by the effect of laser ablation [14]. Chromosome moving was executed with optical tweezers [14] to transfer/isolate the chromosome or fragment of genetic material by the optical trapping effect. The optical tweezers can also perform chromosome stretching and suspension. There were several studies about using laser micro beams to cut and move chromosome or chromosome fragments [14–23], and to alter selectively gene expression by removal of an active genetic region [16] or induce DNA interstrand crosslinks, ablate chromosome telomeres and disturb the mitosis process [21]. However, the previously implemented techniques were lacking in detailed information about the correlation between the parameters of micro beam laser and the incision width and quality upon the cut chromosomes. Moreover, the limited attempts to use laser micro-beams for chromosomal material manipulation have not included directed chromosomal modification to bring about the desired addition, repair of deficient genetic material. Therefore in this paper, we propose the technology of chromosome welding for chromosomal modification.

Laser chromosome welding is the key step for laser chromosome repair and artificial chromosome creation. Laser chromosome welding is a new concept of chromosome micromanipulation and based on the photo-thermal effect of laser to melt the proteins of chromosome so as to form a chromosome bond instantly. It is performed by laser welding to join two adjacent pieces of genetic material. Unlike the tissues in laser tissue welding [24, 25], chromosomes are very small in size and are extremely thin (about 0.5-0.7 μm for human chromosomes) [26]. To weld the two tiny pieces of chromosome together without thermal and mechanical damage upon them is a great challenge in biotechnology and optical engineering. To date there are no reports of such an idea and technology, consequently our method would be a de-novo approach to genetic manipulation.

In this paper, we performed investigations on the technology of chromosome welding, and disclose a novel method of laser chromosomal micromanipulation and modification for chromosome repair and artificial chromosome creation.

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2. Materials & methods

2.1. Chromosomes and reagents

The chromosomes used for our study were derived from fruit fly and B16 cells. The preparation of the salivary gland chromosome of fruit fly (Drosophila melanogaster) was in the similar way described previously [27]. The reason that we used the salivary gland chromosome of a fruit fly for our experiment was that, compared with the chromosomes of most mammal cells, plant cells and fungus, the chromosomes of fruit fly are larger and much longer, allowing for easy observation. Moreover, they have characteristic light and dark banding patterns that can be used to identify chromosomal rearrangements and deletions. On the other hand, studying the genetic makeup, transcription and replication of the fruit fly chromosome can assist in better understanding the processes in other eukaryotic organisms, such as humans. Therefore, we used the chromosomes of fruit fly as the representative chromosome for the study of laser chromosome micromanipulations.

The chromosome of B16 cell was also used in the experiment. The B16 cells were kindly provided by Prof. LX Wang, South East University of China. The cells were cultured in a culture media (89 mL RPMI-1640 + 10 mL fetal bovine serums + 1 mL Penicillin-Streptomycin Solution). Then the cells were treated with 0.9 μg/ml colchicine for 8 h to hold them in the mitosis metaphase. Thereafter, the target cell was held by a micropipette with negative pressure and cut open by a micro beam laser (the laser scissors) to release the chromosomes from the cell. The chromosomes were then isolated by laser tweezers (detailed procedure are described in Results section).

2.2. Experimental set up

Figure 1 is the setup of the P.A.L.M Micro laser Combisystem(P.A.L.M. AG, Germany)for laser chromosome welding, cutting and moving. It was based on a Zeiss inverted microscope and equipped with a pulsed nitrogen laser as the laser scissors and a CW YAG laser as the laser tweezers. The wavelength of the pulsed nitrogen laser was 337 nm. The laser emitted light pulses of pulse energy > 270μJ, pulse duration of 3 ns and pulse frequency of 1-30 per second. The YAG laser (the laser tweezers) worked in a TEMoo mode with wave length of 1064 nm and output power of 1 W. The microscope was also equipped with a two arm multi-joystick robotic micromanipulator to transport the cut chromosome fragments for sequencing or other manipulating purpose such as holding a cell.

2.3. Laser chromosome welding

The laser chromosome welding was performed with a pulsed nitrogen laser micro-beam in the platform of the P.A.L.M Micro laser Combisystem. By using a 63 × oil objective, the diameter of the micro beam was focused to 0.8 μm. The energy used for welding was from 111 to 135 μJ. According to the results of a trial experiment, the energy would just melt the two chromosomes together but without damaging them by combining with proper exposure time. The chromosome welding process requires the combination of laser micro beam cutting, moving and welding. At first, a chosen chromosome fragment was cut with the violet laser micro-beam, and then the fragment was moved by the laser tweezers to the desired location for welding (beside the recipient chromosome). At which the fragment was usually needed to be stretched and smoothed flat by the laser tweezers before welding. Then the fragment was docked and held together with the recipient chromosome. Thereafter the welding of the two chromosome was carried out with a butt welding technique by which the violet laser beam was focused on the bottom side of the chromosomes, and scanned along the join slowly (~0.5μm/s, depending on the energy as mentioned in the Result section) for one to two times.

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2.4. Laser chromosome cutting

The chromosome was cut also with the pulsed nitrogen laser beam but using energy of ≥ 132μJ. The laser beam was focused with either a 63 × or a 100 × oil objective and scanned on the chromosome samples either along a straight line or a curved line. The cutting process can be repeated on a particular target site as needed. The incision width (ΔL) and the quality of the incision were measured as functions of the laser energy.

2.5. Laser chromosome moving and manipulations

In chromosome manipulation and chromosome fragment moving, we used the laser tweezers to transfer the chromosome or fragment of genetic material to the desired location by the effect of optical trapping. The optical tweezers also perform the tasks of chromosome stretching and suspension.

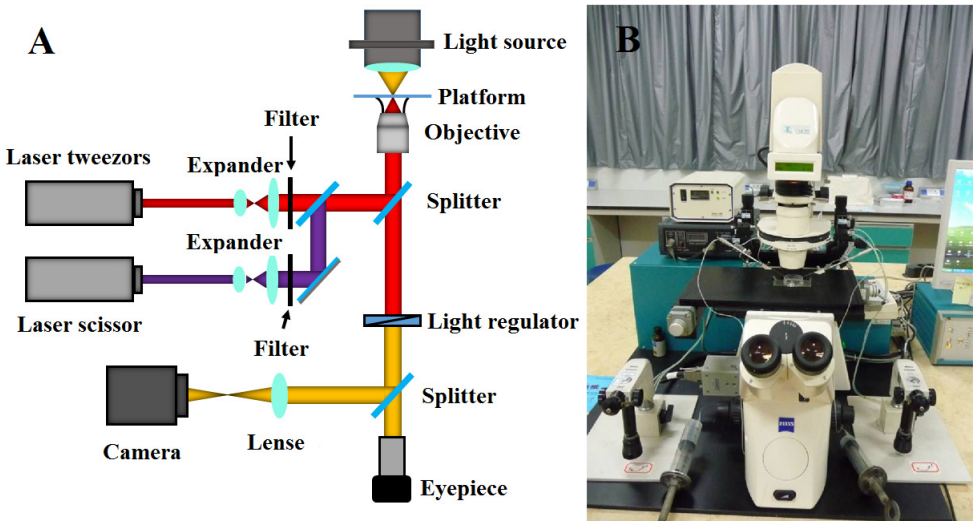

Fig. 1. The structure (A) and the setup (B) of the P.A.L.M Micro laser Combisystem equipped with laser scissors, laser tweezers and micromanipulators.

2.6. Scanning electron microscopy

To test if the laser chromosome welding would have any thermal or mechanical damages on the welded chromosome and if the welded chromosomes were well joined in a solid weld, scanning electron microscopy was performed on the joint of the welded chromosomes using a XL-30E type electron microscope (Philips, Holland). The samples for SEM imaging was prepared with the similar way described previously [28], The SEM image of the chromosome welding joint was then taken at 200 KV with a magnification of 25493.

2.7. Confocal Raman micro-spectroscopy

To test if the welded chromosomes can retain their activity after welding, Raman micro-spectroscopy on the welded chromosomes was also performed with a LabRAM Confocal Raman micro-spectroscope (HORIBA Jobin Yvon, France). The excitation light was 514 nm in wavelength and its output power was 25 mW and the exposure time was 1 s. The excitation light was focused by a 63 × objective at the edge of the welded chromosome near by the welding joint for Raman spectrum measurement.

3. Results

Figure 2 shows a chromosome (salivary gland chromosome) of a fruit fly before and after laser micro-beam cutting (see Fig. 2(A) and (B)). We can see that the incision was cut with a

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very fine degree. Figure 2(C) shows the cutting on a chromosomal puff of fruit fly which was under gene transcription or amplification. Figure 3 illustrates the process of preparing and cutting the chromosomes of a B16 cell. It should be noted that the chromosomes were not stained so that their activity could be retained. Figure 4 shows the determination of the incision width (ΔL) and the variation of ΔL with cutting energy. According to the data shown in Fig. 4, the chromosome could be cut with energy of ≥ 132μJ, and the incision was quite regular and sharp but ΔL increased with cutting energy. By using the energy of 132 μJ, ΔL can be as small as 300 nm. This was quite satisfactory, though we found that ΔL can be even smaller to about 200 nm in a chromosome cutting with a 100 × objective. Therefore, one can choose a suitable energy for a proper cutting on the desired chromosome.

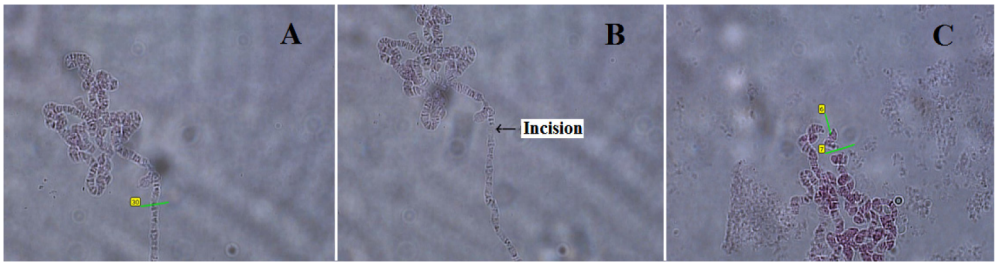

Fig. 2. The laser cutting on a chromosome of fruit fly. (A) selecting the incision part on the chromosome before cutting; (B) the chromosome after cutting; (C) the cutting on a chromosomal puff.

For chromosome moving and manipulation, a wide range of power densities from 10×10^9 W/m^2 to 200×10^9 W/m^2 were found suitable for the task depending on the size of chromosome. In Fig. 3(D), a cut chromosome fragment was moved to a clear environment ready for transporting to a PCR machine for amplification and subsequent sequencing, which was captured by the laser tweezers with a power density of 20×10^9 W/m^2. We had also moved a chromosome group with a power density of 100×10^9 W/m^2 as shown in Fig. 3 in which the chromosome group was released from a target B16 cell which membrane was cut open with a micro beam violet laser (Fig. 3 (B)). The chromosomes were then moved away and separated by the laser tweezers (Fig. 3(C)). From the two instances we can see that since there was no mechanical contact, the processed chromosomes can retain their structure and condition without any mechanical damage.

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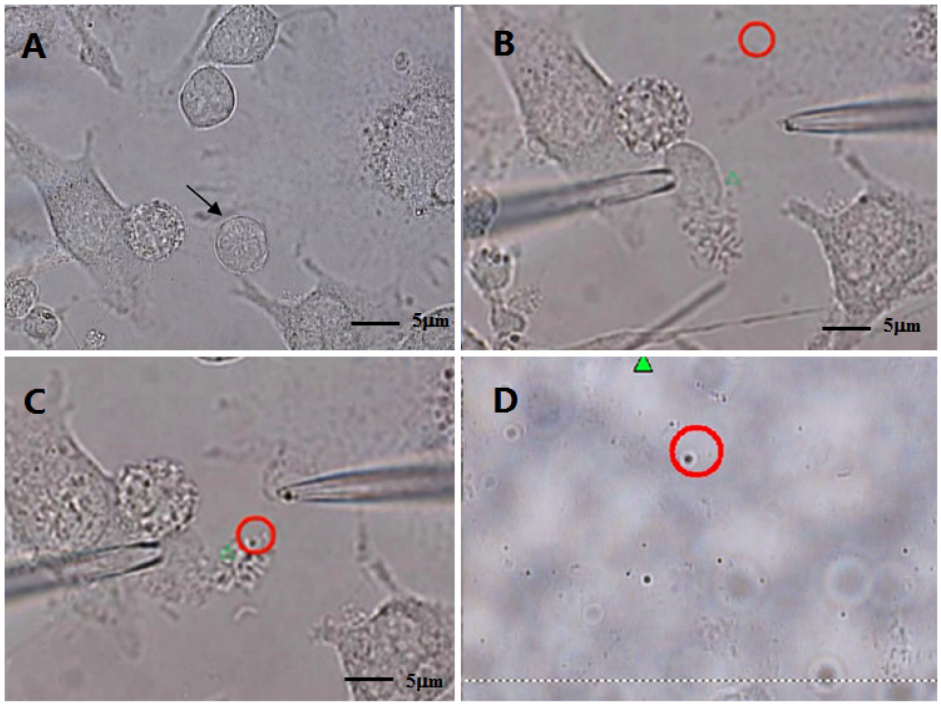

Fig. 3. The preparation and cutting of a B16 cell chromosome. (A) a B16 cell at the mitosis metaphase; (B) The target cell was held by a micropipette with negative pressure and the cell membrane was cut open by a micro beam laser to release the chromosomes from the cell; (C) Chromosome fragment cut off; (D) the cut chromosome fragment was isolated and moved to a clear environment by the laser tweezers.

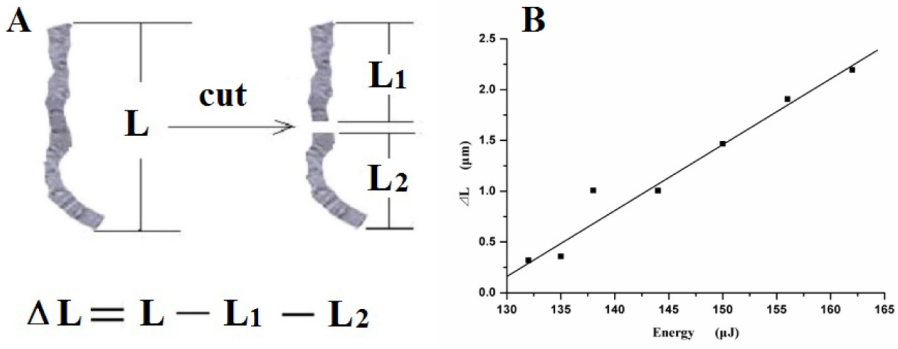

Fig. 4. The incision width (ΔL) and its variation with the cutting energy of laser micro beam. (A) The definition of ΔL: (B) The variation of ΔL with cutting energy.

The purpose of chromosome welding was to repair/replace defective chromosome or chromosome segment and create an artificial chromosome. Figure 5 shows the proper combination of energy and exposure time for chromosome welding. To avoid thermal damage effect of the laser, according to the result of our experience, we recommend using the combination of higher energy and shorter exposure time, such as 130 μJ for 5 seconds.

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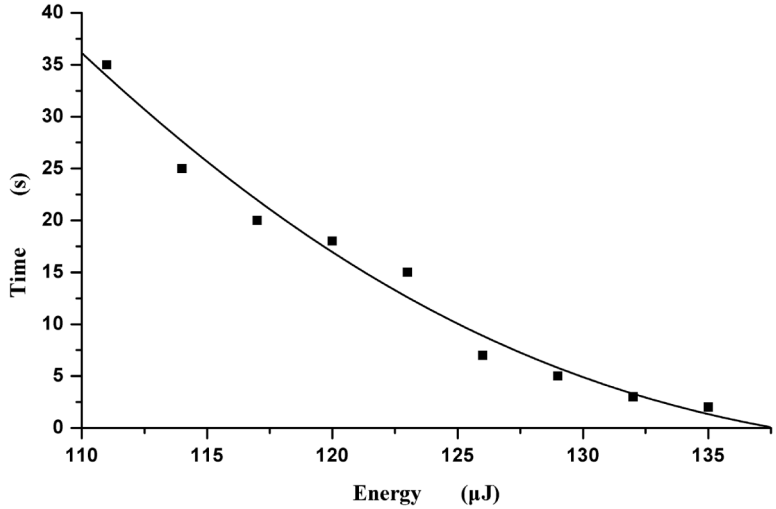

Fig. 5. The suitable combination of exposing energy and time for laser chromosome welding.

Figure 6(A-C) shows the process of cutting a chromosome fragment from a short chromosome of a fruit fly and then moving it with the laser tweezers to a place in the vicinity of a long chromosome of the same cell. The telomere of the second one was removed beforehand and its left part was taken as the recipient chromosome. The end section of the recipient chromosome was manipulated by the laser tweezers to ensure that it suspended in solution but not adhered on the bottom of the culturing dish. The cut chromosome fragment was then stretched and docked with the recipient chromosome by the laser tweezers, and the two adjacent pieces of genetic material were ready for joining by way of laser welding. Thereafter they were welded together by the low energy violet laser micro-beam with a butt welding technique by which the laser was focused on the bottom side of the chromosomes. We can see that the two chromosomes were joined in a solid weld with very fine welding port. Even under a 100 × objective observation (Fig. 6(F)), no gap could be seen in the joint between the two segments. Under direct observation it appeared identical to an unabridged chromosome. The supplementary video gives more detailed information about the entire process of the chromosome cutting-moving-welding (see supplementary Visualization 1). In addition to the similar cutting-moving-welding process as described in Fig. 6, to test the welding effect and the welding strength, the welded chromosomes were heavily swung by the laser tweezers to see if the joining part could be detached by shaking. The result indicated that the two chromosomes were well joined in a solid weld.

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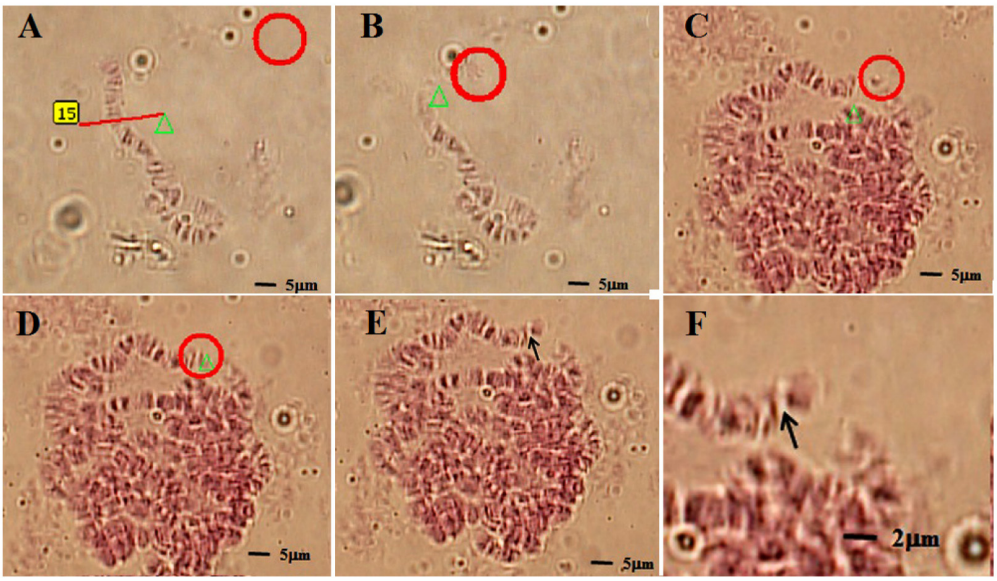

Fig. 6. The process of cutting ((A) and (B)) and moving ((C) and (D)) a chromosome fragment from a short chromosome of a fruit fly and then welding to a long chromosome of the same fruit fly(D). (E) and (F) illustrate the chromosome after welding by 63 × and 100 × objectives respectively.

In addition to the welding strength, the major concerns about the chromosome welding included whether the welding would have any thermal or mechanical damage to the chromosome, and if the chromosomes could retain their activity after welding. To answer these questions, we performed scanning electron microscope (SEM) measurement and confocal Raman micro-spectroscopy on the welded recombinant chromosomes. The reason to use Raman spectroscopy [29] was that the whole chromosome can be studied without physical destruction of the chromatin, thus permitting accurate macromolecular analysis of chromosome structure. Figure 7(A) is the SEM image of the welding joint of the recombinant chromosome. It can be seen that there was a welding joint of about 1μm between the two chromosome fragments, and the welding joint was full of melting material (fused proteins of the chromosome) which connected the two fragments together. The incision was a sharp cut and even in the vicinity of the immediate weld site no burning or impact damage was seen and the two fragments remained their intrinsic structure and integrity. The width of the welding joint was found to depend on the welding parameters and the alignment of the two segments. So it can be envisioned to have a smaller width of the welding joint less than 0.3μm by aligning the two segments better and using a 100 × objective with proper welding parameters. The Raman spectra shown in Fig. 7(B) demonstrated that the welded chromosome did in fact retain DNA activity and integrity, for the bands of 663,726,754, 1248, 1304, 1381, 1423, 1495,1580 cm^{-1} corresponded to bases, while the bands of 797, 832, 939, 1026, 1069, 1342, 1418, 1432, and 1460 cm^{-1} corresponded to deoxyribose. Both the peak location and intensity were basically the same as that of a normal chromosome, indicating that the laser welded recombinant or artificial chromosome had the distinct DNA components and activity that mimic those of natural chromosomes.

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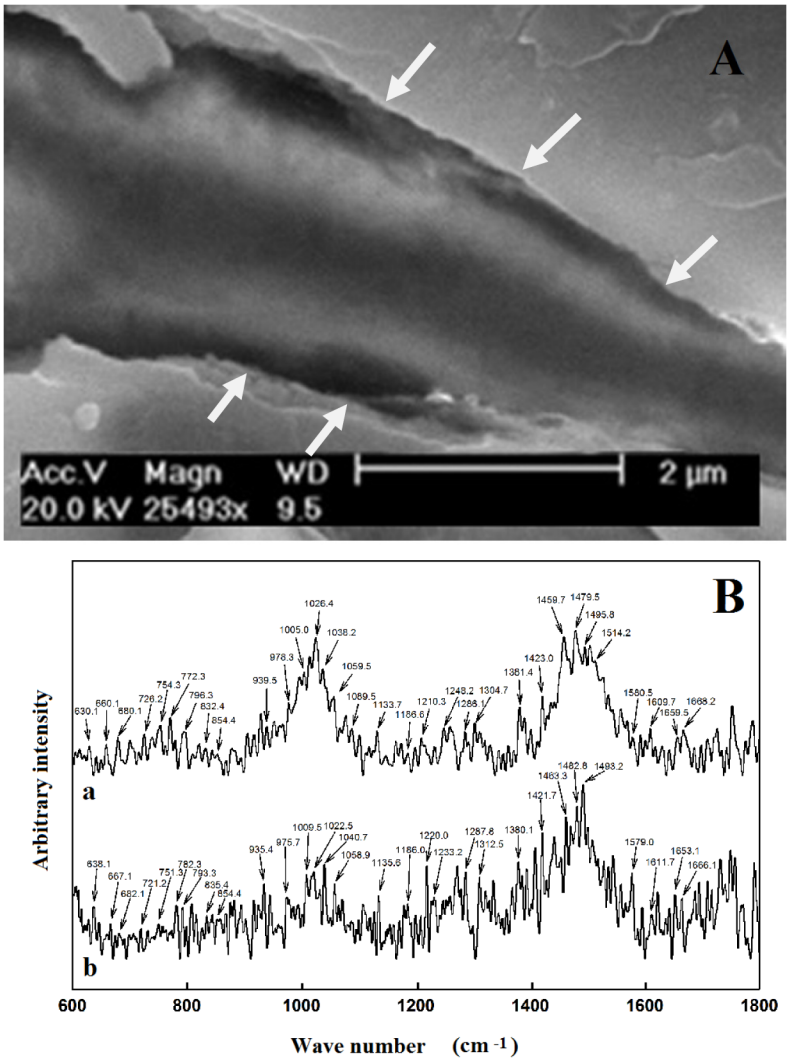

Fig. 7. The SEM image and Raman spectra of the welding joint of a recombinant chromosome. (A) The SEM image of the welding joint of a recombinant chromosome with the white arrows indicating the boundary of the welding join; (B) The Raman spectra taken from the edge of the welding joint of the recombinant chromosome (a) and a native chromosome without any laser manipulation (b).

4. Discussions

We have developed a novel technology of laser chromosome welding which combines the techniques of laser chromosome cutting and moving, and successfully performed the first directed laser chromosome recombination. We demonstrated that with the technology, one can repair/replace defective chromosome regions or even create a recombinant or artificial chromosome containing all the elements required for successful maintenance in vivo, including the telomere, centromere and all the other cis-acting elements with an easier way to a fine degree of control. Our technique has the following advantages and beneficial effects relative to existing technologies: (1) the technique of micro laser beam chromosome welding accompanied with laser chromosome cutting and moving enables people to greatly enhance chromosomal modifications. As a result, it is now easier to create artificial chromosomes by the following strategy: integrate different sizes of chromosome fragments with the target gene

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or any chromosome element into a host chromosome (non-homologous), and repair a chromosome by cutting off its deficient section and then inserting a defect-free region from another homologous chromosome by laser welding. (2) Restriction enzymes, DNA ligases and DNA polymerases are not required for laser cutting or welding of genetic material, greatly simplifying the processing steps, increasing ease of target handling and reducing overall processing costs and time. (3) By the aid of DNA and genomic libraries, the inserted DNA and its size can be exactly defined in the laser chromosome micro manipulations. Using conventional methods, the integration of DNA fragments is a random process and largely uncontrolled [30], and the inserted DNA and its size is not known precisely and has to be tested afterwards by specific primers and assays such as PCR and FISH where only statistically average size can be obtained [31]. (4) No limitation on the size of chromosome fragment for inserting or integrating into a recipient chromosome to replace a defected part of the chromosome or to create an artificial chromosome by the laser chromosome manipulation. Conventional methods have been size limited and large genomic fragments are not successfully integrated into de novo vectors [32, 33]. (5) Genetic material manipulation and chromosome modifications are carried out only with micro-beam laser technology, and as a result, mechanical contact is not required for chromosomal manipulation, so that the processed chromosome can retain its structure and condition without mechanical damage.

The disclosed technique, however, does have limitations. First, since the technique uses an optical microscope to focus the laser, the precision of chromosome cutting and welding is limited to ~200 nm by the diffraction-limited focused laser beam, so the laser chromosome cutting-welding cannot operate with resolution to link up individual base pairs as the enzyme and CRISPR/Cas9 techniques do at the present [6, 7, 10, 34]. Second, during the chromosome cutting and welding, there will be some bases missing due to laser ablation and fusion. Therefore, one should consider such a factor to choose a proper combination of the laser energy and exposure time to have an incision as small as possible and avoid cutting at the region with the desired gene. Finally, the instruments used for the technique are quite expensive so it would limit the broad application of the technique, for the technique needs to use a high power microscope which is coupled with both laser scissors and laser tweezers together as well as other facilities such as multi-joystick robotic micromanipulator and cell culture system.

We are aware of that the work presented in here is just a preliminary exploration on the possibility of applying the technique of laser chromosomal welding for chromosome recombination and artificial chromosome creation using an optical methodology. Although the Raman spectroscopy has proved that welded chromosomes retained their intrinsic structure and integrity, it is insufficient to verify its viability in a recipient cell. In the next step, similar to the way of creating artificial chromosomes by conventional methods, the biological properties and function of the recombinant or artificial chromosomes created by our method should be tested in recipient cells to further prove the validity of the technique. Nevertheless, our work provides a potential novel alternative approach to directed genetic recombination. Even though the technique cannot cut and connect chromosomes as precisely as enzymes do at the present, it would play complementary role in the situations where the conventional methods have difficulty to deal with, such as integrating large genomic fragments into a host chromosome. It may help to generate artificial chromosomes without using restriction enzymes, DNA ligases and DNA polymerases, and can be used for chromosomal repair, removal of defects and creation of new chromosomes. By the aid of DNA and genomic libraries, the technology can realize high efficiency precise cutting and welding on any particular desired genomic fragment to create recombinant or artificial chromosomes with all the necessary elements for successful maintenance in vivo. While the methods are described for use with chromosomes, it is an express intent of the technology that other sort of genetic material such as very large segments of DNA can be manipulated according to the methods. And not limited to the genetic material or chromosomes of fruit

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flies, but may also include all kind of humans, animals, plants and micro-chromosomal fragments as well as synthetic chromosomes.

Funding

National Natural Science Foundation of China (Nos. 30940019 and 60377043); Guang Dong Provincial Science and Technology Foundation (No.2015B010105006 and 2013B060100011); Guang Zhou Science and Technology Foundation (No.2014Y2-00508 and 33116013).

Acknowledgments

We would also like to gratefully acknowledge support from the GeneForge Ltd and J Schroeder.

YXH conceived and designed the experiments. L L, L Y and Y Zh performed the experiments. YXH, analyzed the data and wrote the paper.

Disclosure

The authors declare a patent on a part of this work has been filed by Ji Nan University with YX H as inventor.